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14. ABSTRACT

Genomic alterations of chromosome 18q have been observed in prostate cancer. This research focuses on analyzing the role of increased gene copy number at chromosome 18q22.1 in prostate cancer. We believe the key genes in this region are the type II cadherins. We are studying the role of overexpression of these genes, particularly cadherin 7 (CDH7), on the tumorigenicand invasive potential of prostate cancer cells. We have shown the minimal region of increased copy number contains CDH7. This increased copy number of CDH7 is specific to prostate cancer and is not found in 11 other common cancers. The increased copy number of CDH7 also results in increased levels of cadherin-7 mRNA in prostate tumors. We are purifying polyclonal antibodies against cadherin-7 to use in immunohistochemistry experiments to determine if increased CDH7 copies results in increased levels of protein. We performed knockdown experiments of the cadherin-7 mRNA in a prostate cancer cell line and are analyzingthe cadherin-7 protein levels in these cells. We will subsequently evaluate these cellswith reduced cadherin-7 expression for their invasive and tumorigenic potential.

15. SUBJECT TERMS
prostate cancer, cadherin, chromosome 18, copy number alteration

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Table of Contents

Introduction

Genomic alterations of chromosome 18q have been observed in prostate cancer. This research focuses on analyzing the role of gene amplification on chromosome 18q22.1 in prostate cancer and the potential function of the critical genes that are found to be present in increased copy numbers. This is innovative research in that we are the first to observe increased copy numbers of genes in this region on chromosome 18 in prostate cancer. We believe the key genes in this amplicon are a class of cell adhesion molecules, the type II mesenchymal cadherins. We are studying the role of overexpression of these genes normally expressed in mesenchymal cells, particularly cadherin 7 (*CDH7*), on the tumorigenic and invasive potential of prostate cancer epithelial cells. These cadherins have never been implicated in prostate cancer, despite the fact that one of the proteins is only expressed in brain, testes and prostate. We will analyze the expression of a variety of cadherin family members in prostate tumors of varying stages and grades. The results of the study will be able to show if overexpression of these mesenchymal-type adhesion molecules can be correlated with more aggressive prostate tumors. This could lead to the development of biomarkers to assess the metastatic potential of a primary tumor.

Body

The research accomplishments for:

Task 1: Identify the smallest common region of amplification on chromosome 18q22.1 in prostate cancer.

a. Perform fluorescence in situ hybridization (FISH) on paraffin-embedded prostate tumor specimens using bacterial artificial chromosomes (BACs) spanning the amplified region.

We previously assembled a complete contig of BAC clones that spanned the original region of altered copy number defined by array comparative genomic hybridization (array CGH). Prostate tumors with increased copy numbers of this region we analyzed by FISH methodologies using hybridization probes created by fluorescently-labeling DNA isolated from two BAC clones that flank the *CDH7* gene. The results from this experiment indicate that the smallest region of altered copy number is a 680 kb region (Figure 1). Located within this region is the gene for *CDH7.* A large part of the coding sequence for this gene is found in the chromosome 18 BAC clone RPCI11-775G23.

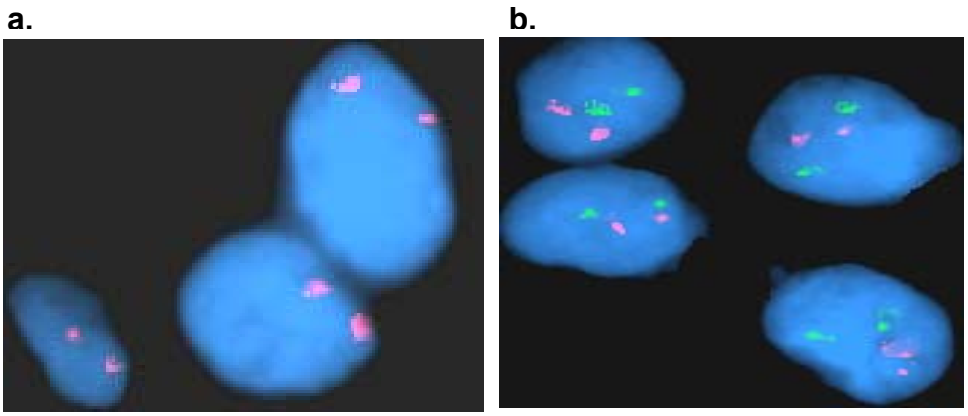

Figure 1: Identification of minimal region of amplification at chromosome 18q22.1. A probe proximal to RPCI11-775G23, RPCI11-453M23 (Spectrum Orange) is present in two copies in both tumor (a) and normal prostate tissues (b), while a distal probe RPCI11-425M2 (FITC) is deleted in the prostate tumor section (a). These two probes are present approximately 680 kb apart from each other on the chromosome.

b. Perform quantitative PCR on genomic DNA isolated from prostate tumor specimens.

Since the *CDH7* gene is located within this region of increased copy number and appears to be present in multiple copies by FISH analyses, we subsequently analyzed the copy number of *CDH7* in prostate tumors using quantitative PCR. DNA was isolated from microdissected prostate tumors showing increased copy number at 18q22.1 and a quantitative assay to measure *CDH7* gene copy number using real-time PCR was developed. The copy number of the *CDH7* gene in the prostate tumors ranged between two and seven (data not shown). For the majority of samples, the gene copy number of *CDH7,* as detected by quantitative PCR, correlated with the degree of amplification of the region homologous to RP11-775G23 detected by array CGH in the corresponding tumor section.

c. Perform quantitative reverse transcription-PCR on RNA isolated from prostate tumors to verify increased gene expression with increased gene copy number.

We tested RNA extracted from microdissected prostate tumors to verify whether the genomic amplification of the gene has any impact at the transcriptional level of *CDH7*. As expected, we detected three- to eight-fold overexpression of *CDH7* mRNA in prostate tumors compared to the normal adjacent tissue (Figure. 2). The cell line PZ-HPV-7, derived from prostate epithelial cells, showed very low *CDH7* expression. The expression of *CDH7* was several hundred-fold higher in the tumors, compared to PZ-HPV-7 (Figure. 2). Together, array CGH, FISH and real-time quantitative PCR show increased copy number of the *CDH7* gene in prostate cancer which correlates with increased expression of *CDH7*.

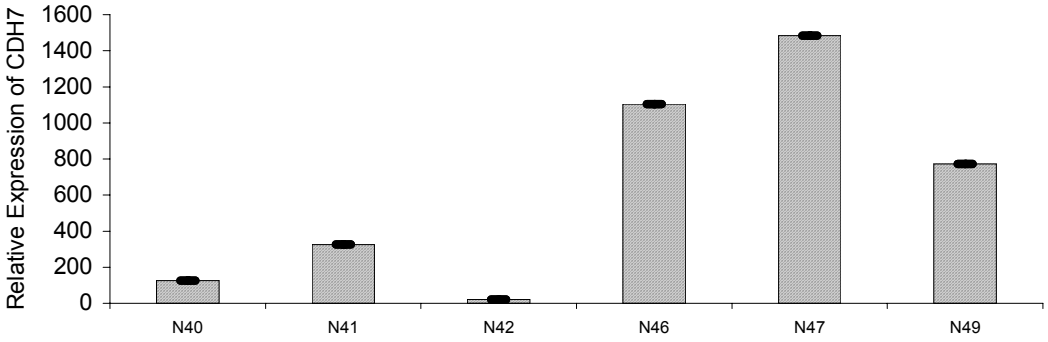

Figure 2: Expression of *CDH7* mRNA as det4ermined by quantitative reverse transcription-PCR.
CDH7 mRNA levels are shown for six prostate tumors relative to the expression of *CDH7* in the prostate epithelial cell line PZ-HPV-7.

Task 2: Investigate the expression of E-cadherin, N-cadherin, cadherin-7, cadherin-11, cadherin-19 and cadherin-20 in prostate tumors of varying stages and grades.

We are investigating the expression of E-cadherin, N-cadherin and cadherin-11 in prostate tumors because E-cadherin has been found to be down-regulated in prostate tumors (Rubin et al., 2001) and co-expression of two of the mesenchymal cadherins N-cadherin and cadherin-11 have been reported in prostate cancer samples (Tomita et al., 2000). We are studying the

expression of cadherin-7 and -19, because the *CDH7* gene is within our minimal region of increased copy number and the cadherin-19 gene is approximately 700 kb distal to the *CDH7.* The gene for another mesenchymal cadherin, cadherin-20 is located 5 megabases proximal to *CDH7* and does not appear to be in increased copy number, but its expression in prostate cancer needs to be assessed. This study will give a more complete picture of changes in cadherin expression that occur during prostate cancer progression.

a. Create polyclonal antibodies to specifically recognize three of the type II cadherins (cadherin-7, -19 and -20.

In order to analyze the expression of the type II cadherin family members cadherin-7, -19 and -20 in prostate tumors of varying stages and grades, we contracted with Sigma-Genosys to create two custom polyclonal antibodies for each of the type II cadherins using unique peptides from the extracellular domain and the COOH-terminus. We received the antisera and performed some preliminary characterization of the antibodies. Our initial results showed that the antibodies directed against the extracellular domain peptides appeared to recognize the appropriate size of protein from cell lysates, as detected by western blotting. However, prior to their use in immunohistochemistry experiments, the antibodies needs further purification. In order to purify the antisera, we have created bacterially-expressed glutathione-S-transferase (GST) fusion proteins that will be used to capture the anti-cadherin antibodies in the antisera. We have bacterially expressed cadherin-7 and experiments are underway to purify the serum antibodies (Figure 3a). In addition we have determined that the anti-cadherin 7 antibody detects cadherin-7 specifically and that antibodies directed against the closely-related cadherins, cadherin-19 and cadherin-20 do not recognize GST-cadherin-7 (Figure 3b).

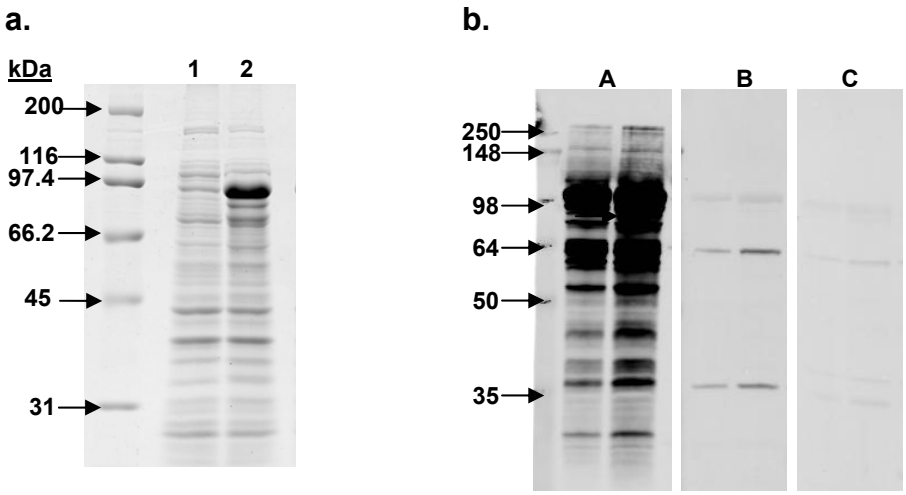

Figure 3. Bacterial expression of GST-cadherin-7
a) Coomassie-stained gel showing GST-cadherin-7 expression in a crude bacterial lysate. Lane 1, uninduced culture. Lane 2, culture after 3h of induction with IPTG. Expected molecular weight of GST-cadherin-7 fusion protein, ~98 kDa. b) Specificity of cadherin-7 polyclonal antibody. Each panel contains a pair of lanes with 5 and 10 ug of crude bacterial lysate expressing the GST-cadherin-7 fusion protein. Panel A was immunoblotted with the crude serum of a rabbit immunized with a small cadherin-7 peptide. Panels B and C were immunoblotted with the crude serum from a rabbits immunized with a small cadherin-19 peptide or cadherin-20 peptide, respectively. The smaller, intense bands in Panel A are degradation products of the fusion protein.

We are also in the process of purifying the polyclonal antibodies against cadherin-19 and cadherin-20 using bacterially expressed maltose binding protein (MBP) fusion proteins (Figure 4). Our initial analysis of the cadherin-19 antibody shows that it specifically recognizes MBP-cadherin-19 and that antibodies against cadherin-7 or cadherin-20 do not recognize MBP-cadherin-19 (Figure 4).

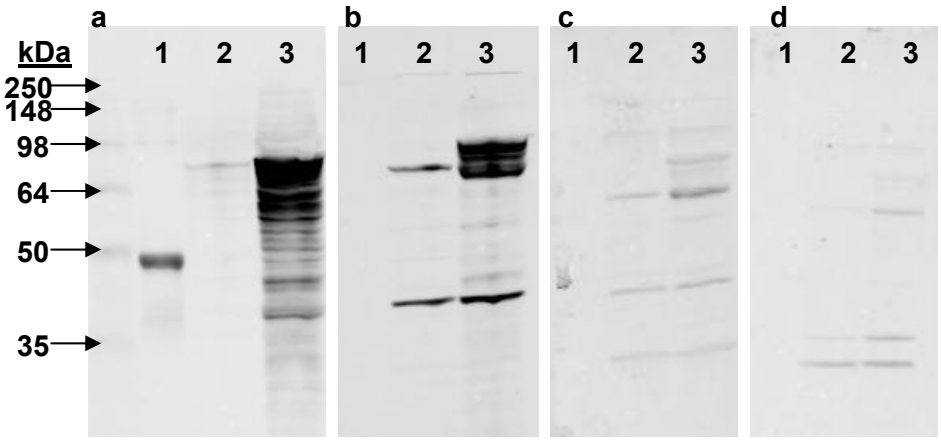

Figure 4. Immunoblot for MBP-cadherin 19 expression in a crude bacterial lysate. Each panel contains the following: maltose binding protein (MBP) (Lane 1), uninduced culture (lane 2) and culture after 3h of induction with IPTG (Lane 3). The expected molecular weight of MBP-cadherin 19 fusion protein is ~72 kDa. Panel a was immunoblotted with anti-MBP and the remaining panels were immunoblotted with the crude serum from a rabbit immunized with a small cadherin 19 peptide (Panel b), small cadherin-20 peptide (Panel c) and small cadherin-7 peptide (Panel d).

b. Prepare tissue microarrays using prostate cancer specimens of various stages and grades.

Imgenex (San Diego, CA) is a commercial supplier of tissue microarrays and they have developed a prostate tumor microarray consisting of 40 tumors or various stages and Gleason grades with matched normal tissue. FISH was performed on this microarray using a hybridization probe derived from the BAC clone RPCI11-775G23 that contains the *CDH7* gene. All 40 prostate tumors showed three or more signals from the RPCI11-775G23 probe (data not shown). The control probe, a centromeric probe from chromosome 18, showed one to two signals in all 40 tumors on the array indicating that chromosome 18 was not completely amplified in the tumors. These results were consistent with our original array CGH data which showed no correlation between *CDH7* copy number and Gleason score (Table 1).

Table 1. Prostate tumor samples with various pathological grades showing amplification at chromosome 18q22.1

Tumor	Age	Gleason	Stage	Amplification at 18q22.1
N10	66	6	T2CNXMX	Medium*
N12	64	6	T2CNXMX	Medium
N15	72	7	T2CNXMX	Medium
N22	59	7	T2CN0MX	High
N26	56	6	T2CNXMX	Low
N30	73	5	T2CNXMX	Medium
N31	72	6	T2CNXMX	Medium
N32	54	8	T2CN0MX	Low
N34	52	7	T3BN0MX	Normal
N35	56	7	T2CNXMX	Low
N36	62	6	T2CNXMX	Medium
N37	62	9	T3AN0MX	High
N38	59	6	T2CNXMX	Medium
N39	60	7	T3NXMX	Low
N40	70	7	T2CNXMX	High
N41	67	7	T3BN0MX	High
N42	67	8	T2CN0MX	Low
N43	65	5	T2BNXMX	Low
N44	53	7	T2BNXMX	Medium
N45	55	9	T2BN0MX	Low
N47	71	9	T3BN0MX	Low
N49	56	9	T2CNXMX	Low

* Log_2 ratio 1-1.5: Low; Log_2 ratio 1.5-2.0: Medium; Log_2 ratio >2.0: High

c. Perform immunohistochemistry experiments to analyze expression of E-cadherin, N-cadherin and the type II cadherins, cadherin-7, -11, -19 and -20 using tissue microarrays.

We are currently purifying the polyclonal antibodies that will detect cadherin-7, -19 and -20. Upon completion of Task 2a, we will be able to proceed with this Task and determine if the increase in copy number of the chromosome 18q22.1 region containing the *CDH7* gene results in an increase in protein in the prostate tumors included on the Imgenex prostate cancer tissue microarray. Antibodies to E-cadherin, N-cadherin and cadherin-11 are commercially-available and the amount of protein expression of these cadherins will also be analyzed to provide a more complete picture of the cadherin expression changes occurring during the tumorigenic process.

d. Perform immunohistochemistry experiments to test the tissue specificity of the three type II cadherins (cadherin-7, -19 and –20).

We have recently performed FISH experiments with the RPCI11-775G23 (contains *CDH7*) probe on tissue microarrays consisting of cancer from 12 organ sites (Imgenex common cancers A and B) The gene copy number alteration detected on chromosome 18q22.1 using probe RPCI11-775G23 on prostate tumor samples was not observed in any other tumors samples, including stomach, esophagus, lung, colon, thyroid, kidney, breast, liver, urinary bladder, ovary and pancreas. These data indicate that the increased copy number of the RPCI11-775G23 region containing *CDH7* is tumor specific, and is limited to the prostate. Of these 12 tissues analyzed, only prostate is known to express *CDH7* (Kools et al., 2000).

Task 3: Knockdown expression of type II cadherins in prostate cancer cell lines and analyze the phenotype of the cells for invasive and tumorigenic potential.

a. Create transient and stable RNA interference constructs and perform experiments to knockdown expression of cadherin-7, -19 or -20, individually.

We have designed short hairpin RNAs (shRNA) for cadherin-7, -19 and -20 using design tools provided by Ambion (The Woodlands, TX). The shRNA was cloned into the pSilencer vector (Ambion) which contains a mammalian selectable marker for creating RNA interference constructs that can be stably selected in a mammalian cell line. The shRNA construct for cadherin-7 was stably transfected into the 22Rv1 prostate cancer cell line, a cell line that expresses cadherin-7. Preliminary analysis of the cadherin-7 mRNA in the stable transfectants shows dramatic reduction in the mRNA levels, but these results need to be confirmed by performing western blots to determine the levels of cadherin-7 protein.

b. Analyze the transformed metastatic phenotype of the prostate cancer cells after knockdown of the type II cadherins using *in vitro* assays for anchorage independence and migration/invasion.

c. Analyze the *in vivo* tumorigenic phenotype of the prostate cancer cells after knockdown of type II cadherin mRNA.

No work to date has been accomplished on Tasks 3b or 3c. These experiments will be performed after we have shown a successful knockdown of the cadherin protein in the 22 Rv1 cells stably transfected with shRNA.

Key Research Accomplishments

- **Defined a minimal region of increased copy number (680kb) of chromosome 18q22.1 containing the cadherin 7 gene in prostate cancer.**
- **Determined that increased copy number of the cadherin-7 gene results in increased cadherin-7 mRNA levels in prostate tumors**
- **Determined that increased copy numbers of the cadherin-7 gene do not correlate with the stage or Gleason score of prostate tumors.**
- **Determined that increased copy number of the cadherin-7 gene is found specifically in prostate cancer and not 11 other common cancers.**
- **Created polyclonal antibodies to cadherins-7, -19 and -20.**
- **Knocked down expression of the cadherin-7 gene in the prostate cancer cell line 22Rv1.**

Reportable Outcomes

We submitted a manuscript to Genes, Chromosome and Cancer describing our results to date and are in the process of resubmitting a revised manuscript for review (See appendix).

Conclusions

We have generated data that show that the minimal region of increased copy number in prostate cancer at chromosome 18q22.1, originally detected by array CGH, is a 680kb region that contains the cadherin-7 gene. This increased copy number of the cadherin-7 gene is specific to prostate cancer and is not found in 11 other common cancers. The increased copy number of the cadherin-7 gene also results in increased levels of cadherin-7 mRNA in prostate tumors. We are in the process of purifying polyclonal antibodies to cadherin-7 to use in

immunohistochemistry experiments to determine if the increase in gene copies affects the level of protein. We have performed knockdown experiments of the cadherin-7 mRNA in a prostate cancer cell line and we will be analyzing the cadherin-7 protein levels in these cells. We will subsequently evaluate these cells lines with reduced cadherin-7 expression for invasive and tumorigenic potential.

We have not found that increased copy number of the cadherin-7 gene is correlated with the stage or Gleason score of the prostate tumors and may be an early marker of prostate cancer. Since the cadherins are a class of cell adhesion molecules and the type II cadherins are mesenchymal cadherins not normally expressed in epithelial cells, the increased expression of cadherin-7 may be a marker of the tumorigenic phenotype. Assays to detect cadherin-7 in prostate tissue have the potential to be developed into clinically-useful biomarkers for prostate cancer.

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Rubin MA, Mucci NR, Figurski J, Fecko A, Pienta KJ, Day ML (2001) E-cadherin expression in prostate cancer: a broad survey using high-density tissue microarray technology. Human Path 32:690-697.

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Appendix
Manuscript submitted to Genes, Chromosomes, and Cancer

Identification of a recurrent amplification on chromosome 18q22.1 containing the cadherin 7 gene in prostate cancer

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Abbreviated Title: Amplification of cadherin 7 in prostate cancer

Abstract

Prostate cancer is the most common non-dermatological cancer of U.S. men. After decades of intense efforts, the molecular etiology of prostate cancer remains poorly understood. Recent advances in technology provide greater efficiency and precision for the identification of alterations in chromosomal regions. Array comparative genomic hybridization is one methodology that has wide applicability in a variety of genomic disorders, including cancer. Using a high-resolution BAC genomic microarray for chromosome 18q21-q23, we have analyzed DNA copy number changes in prostate cancer. With this array, a novel amplification on chromosome 18q was found in approximately 55% (12/22) of primary prostate cancers. The maximum amplicon was a region of approximately 3.8 Mb. By fluorescence in situ hybridization (FISH) analysis of tumors, the minimal region of amplification was estimated to be less than 680 kb. This region contains the cadherin 7 gene (*CDH7*) and no other known genes. Tissue arrays containing 12 different cancers were evaluated by FISH, and amplification of *CDH7* appears unique for prostate cancer. Increased *CDH7* gene copy numbers, as well as increased mRNA levels, were demonstrated by quantitative PCR. Our data support a role for *CDH7* in the development of prostate cancer.

Introduction

The chromosomal aberrations observed in prostate cancer are numerous, but few are implicated in the progression to metastatic disease (reviewed in Porkka and Visakorpi, 2004). Previous studies using comparative genomic hybridization (CGH) showed that chromosome 18q is frequently lost in prostate cancer (Brothman et al., 1999). In addition, allelic imbalance (AI) on chromosome 18q is observed more frequently in metastatic prostate cancer (Ueda et al., 1997; Padalecki et al., 2000). However, amplification of chromosome 18q, at lower frequency, has also been observed in prostate cancer (Nupponen et al., 1998) and other cancer types (Monni et al., 1997; Yin et al., 2000). Increased copy number of *BCL2*, an anti-apoptotic gene located on 18q, is rarely observed in these cancers, indicating that there are other targets of amplification at 18q.

Novel candidate genes have been identified using array CGH in other cancers (Redon et al., 2002). Bacterial artificial chromosome (BAC) microarray (Pettus et al,. 2004) and cDNA microarray (Clark et al., 2003) have been used to detect DNA copy number changes in genome-wide analyses on prostate cancer. However, the average resolution of whole genome BAC microarrays used in earlier studies to identify genetic changes in prostate cancer was approximately 1 Mb (Pettus et al., 2004). These arrays are less capable of detecting single BAC clone changes from a specific chromosomal segment, as compared to arrays constructed with genomic clones from the tiling path. To characterize distal chromosome 18q for copy number variations, we have developed a high-resolution tiling path resolution BAC microarray spanning 18q21 to 18q23. This microarray identified a novel chromosomal amplification on 18q in primary prostate cancer.

Materials and Methods

Tumor samples

All studies involving human samples were approved by the Institutional Review Board at the University of Texas Health Science Center at San Antonio. The prostate tumor samples were from patients who had undergone radical prostatectomy at the University Hospital, San Antonio, TX and Audie Murphy

Veterans Administration Hospital, San Antonio, TX. These were formalin-fixed paraffin-embedded tissues processed and stained by standard histological techniques.

Cell lines

The cell line PZ-HPV-7 was purchased from American Type Culture Collection (ATCC) and grown in the recommended medium and culture conditions.

DNA extraction

DNA was extracted from four 10 µm tissue sections of paraffin-embedded specimens as previously described (Vijayakumar et al. 2006). Briefly, the tumor tissue was microdissected from these sections using a corresponding H&E stained slide as the template. After dewaxing using xylene, tumor tissue was digested in lysis buffer (10 mM Tris-HCl, pH 8, 0.5% SDS and 20 µg/ml RNAse) containing 20 µg/ml Proteinase K (Roche Diagnostics, Indianapolis, IN). Once the digestion was completed, DNA was extracted with phenol/chloroform. The DNA pellet was resuspended in 50-100 µl of sterile water, quantified with a fluorometer and quality tested by electrophoresis through a 1% agarose gel.

Array CGH

From the chromosome 18 tiling path (UCSC genome browser, 2001-2003 freezes), 165 BAC clones were selected and PCR-verified. The BAC DNA was extracted using the Plasmid Maxi kit (Qiagen, Valencia, CA) and spotted onto slides in duplicate by Spectral Genomics (Houston, TX). The slide format was based on the company's Constitutional Chip^{TM} array. One µg each of tumor DNA and normal male DNA (Promega, San Luis Obispo, CA) was sonicated (Sonic Dismembrator, Fischer Scientific, Pittsburgh, PA) and labeled with Cy5 and Cy3 (Perkin-Elmer, Boston, MA) using the Bioprime DNA labeling system (Invitrogen, Carlsbad, CA). The labeling, hybridization and washing of the slides were done as recommended by Spectral Genomics, except that the washes were carried out at 42°C (Vijayakumar et al. 2006). The data processing of Cy5/Cy3 ratios was accomplished using SpectralWare, version 2.1 (Spectral Genomics).

Fluorescence *In Situ* Hybridization

For normal metaphase FISH, a primary peripheral blood culture from a normal male donor was used. For paraffin-embedded tissue (5 µm thickness), the paraffin pretreatment reagent kit II (Vysis Inc., Downers Grove, IL) was used, according to the manufacturer's protocol. Amplification of the signal was performed on both the normal metaphase and paraffin-embedded tissue. A previously described protocol for FISH was used for performing the hybridization (Padalecki et al., 2001). The DNAs from BAC clones RP11-453M23, RP11-425M2, RP11-775G23 and RP11-389J22 were used as probes. A centromeric probe specific for human chromosome 18 (Vysis Inc., Downers Grove, IL) was used as the control. Four,6-diamidino-2-phenylindole (DAPI) was used as the counter stain. Slides were viewed using a Zeiss Axioplan 2 fluorescence microscope and the images captured using Genus software (Applied Imaging, San Jose, CA).*FISH on human tissue arrays*: Two different types of tissue arrays were purchased from Imgenex (San Diego, CA). One set contained 40 prostate tumors at various grades and stages (slide CA3), The other set contained a total of 12 common tumors (slides MA2 &MB2) from the stomach, esophagus, lung, colon, thyroid, kidney, breast, liver, urinary bladder, ovary, pancreas and prostate. The matched normal samples from these 12 tumors (slides MA2 &MB2) were also used in the analyses. The protocol used for FISH was essentially the same as the one used for paraffin-embedded tissue sections.

Real-time quantitative PCR

The TaqMan primer-probe sets were designed using Primer Express 2 software (Applied Biosystems, Foster City, CA).

Analysis on genomic DNA: For testing DNA copy number of *CDH7*, the primer sequences used were,

CDH7A: 5'CAAAGACAACACAGCCTCAATACTG3'-Forward

CDH7B: 5'TGGCAGATAGTAAACTGATTGTTCCT3'-Reverse

The primer sequences for the internal control (Goff et al. 2000) were,

Albumin*: 5'AGGGTAAAGAGTCGTCGATATGCT3'-Forward*

Albumin*: 5'CAATCTCAACCCACTGTCAGCTA-Reverse*

The TaqMan probes used were, *6FAMTCCGGAAGCCGTTTCTCCTGGTAMRA* and *6FAMCAAACGCATCCATTCTACCAACTTGAGCATTAMRA* for *CDH7* and albumin, respectively.

Expression Analysis: For real-time RT PCR, the primer sequences used for *CDH7* were,

CDH7B: 5'TGGCAGATAGTAAACTGATTGTTCCT3'-Reverse

CDH7C: 5'CAACAAATAACCACAACTTTTCATTG3'-Forward

The same *CDH7* probe was used to test both the DNA copy number and the expression levels of *CDH7* mRNA. For the internal control, the TATA-box binding protein expression assay (Applied Biosystems, Foster City, CA) was used in gene expression analysis.

All reactions were conducted in 384-well plates using the 7900HT Sequence Detection System (Applied Biosystems, Foster City, CA) following the protocol recommended by the manufacturer. TRIzol reagent (Invitrogen, Carlsbad, CA) was used to extract total RNA from frozen sections of the tumor. First strand cDNA was synthesized from these RNAs using the TaqMan Reverse Transcription kit (Applied Biosystems, Foster City, CA) and were used as templates in reactions consisting of 1X TaqMan Universal PCR Master mix (Applied Biosystems, Foster City, CA), 900 nM of *CDH7*B/*CDH7*C primers and 200 nM *CDH7* probe. For the endogenous control TATA-box binding protein (*TBP*) expression assay (Applied Biosystems, Foster City, CA) was used. To test DNA copy number, 20 ng of genomic DNA, 900 nM of *CDH7*A/*CDH7*B primers and 200 nM probe were used for *CDH7*; the control albumin had similar conditions except that the albumin primers were used at a concentration of 300 nM each. The PCR cycling conditions consisted of 50° C for 2 min; 95° C for 10 min; and 40 cycles each of 95° C for 15 sec and 60° C for 1 min. The results were analyzed using the SDS software (Applied Biosystems, Foster City, CA).

Results

Frequent amplification at 18q22.1 detected by array CGH

The microarray we constructed contained 165 PCR-verified chromosome 18q BAC clones which were used to analyze 22 primary prostate tumors. The most prominent copy number alteration was an amplification at 18q22.1, which was observed in 55% of prostate tumors (12/22) (Fig. 1). The amplicon size varied between samples, with the maximum amplification spanning the chromosome 18q region contained in BACs RP11-775G23 through RP11-543H23 (approximately 3.8

Mb). There are four known genes, cadherin 7 (*CDH7)*, cadherin 19 (*CDH19)*, thioredoxin domain containing 10 *(TXNDC10*) and docking protein 5-like *(DOK6)* present in this amplified region. The highest level of amplification observed on chromosome 18 corresponded to the region homologous to BAC clone, RP11-775G23 (Fig. 1), which contains the 5' end of *CDH7*.

Verification of amplification at 18q22.1

Since the most striking copy number variation was observed with the BAC RP11-775G23, we further verified the amplification detected by array CGH using fluorescence in situ hybridization (FISH) and real-time quantitative PCR. We tested two prostate tumors that exhibited high-level amplification, specimens N40 and N41, using FISH with a probe derived from BAC clone RP11-775G23 and a probe derived from the adjacent BAC clone RP11-389J22, which contains the majority of the *CDH7* gene. Both tumors showed more than the normal two copies of the genome contained within BAC clones RP11-775G23 and RP11-389J22 (Fig. 2). Furthermore, the amplification was tumor-specific because the adjacent normal cells contained only two copies of these regions. Two additional probes derived from BAC clones flanking RP11-775G23, RP11-453M23 and RP11-425M2, were used for FISH on tumor N41 to further narrow the region of amplification (Fig. 3). Two copies of the genome contained within RP11-453M23, present approximately 350 kb proximal to RP11-775G23, were present in all nuclei scored from N41. We did not detect the region homologous to clone RP11-425M2, approximately 330 kb distal to RP11-75G23, in the N41 tumor cells, while two copies of the RP11-425M2 region were present in the adjacent normal tissue. The BACs RP11-453M23 and RP11-425M2 are approximately 680 kb apart, thus the minimal region of amplification is estimated to be less than 680 kb.

To determine if this genetic alteration was correlated with tumor stage and grade, a prostate cancer tissue array consisting of 40 tumors of various stages and Gleason scores was analyzed with FISH using the probe derived from BAC clone RP11-775G23. All 40 prostate tumors showed three or more signals from the RP11-775G23 probe (data not shown). The control probe, a centromeric probe from chromosome 18, showed one to two signals in all 40 tumors on the array indicating that chromosome 18 was not completely amplified in the tumors. These results were consistent with our array CGH data which showed no correlation between *CDH7* copy number and Gleason score (Table 1).

The amplification detected on chromosome 18q22.1 using probe RP11-775G23 on prostate tumor samples was not observed in any other tumors samples, including stomach, esophagus, lung, colon, thyroid, kidney, breast, liver, urinary bladder, ovary and pancreas. These data indicate that the amplification of RP11-775G23 is tumor specific, and is limited to the prostate. Of these 12 tissues analyzed, only prostate is known to express *CDH7* (Kools et al., 2000).

To quantitate the fold difference in the gene copy number of *CDH7* between the tumor and normal cells, real-time quantitative PCR was performed on genomic DNA isolated from the tumors that were used for array CGH analyses. The copy number of the *CDH7* gene in the prostate tumors ranged between two and seven. For the majority of samples, the gene copy number of *CDH7,* as detected by quantitative PCR, correlated with the degree of amplification of the region homologous to RP11-775G23 detected by array CGH in the corresponding tumor section. These findings are consistent with the hypothesis that *CDH7* is a key target for amplification in prostate cancer.

Quantification of *CDH7* mRNA

We tested RNA extracted from microdissected prostate tumors to verify whether the genomic amplification of the gene has any impact at the transcriptional level of *CDH7*. As expected, we detected three- to eight-fold overexpression of *CDH7* mRNA in prostate tumors compared to the normal adjacent tissue (Fig. 4). The cell line PZ-HPV-7, derived from prostate epithelial cells, showed very low *CDH7* expression. The expression of *CDH7* was several hundred-fold higher in the tumors, compared to PZ-HPV-7 (Fig. 4). Together, array CGH, FISH and real-time quantitative PCR show amplification of *CDH7* gene in prostate cancer which correlates with increased expression of *CDH7*.

Discussion

Using a tiling path BAC array, we describe a novel amplification on chromosome 18q22.1 and show that the most likely candidate gene involved in this amplification is *CDH7*. Using FISH and quantitative RT-PCR, our study reports frequent amplification and overexpression of *CDH7* in primary prostate cancer. The cadherins are a large family of transmembrane glycoproteins belonging to the immunoglobulin superfamily that mediate cell-cell adhesion and communication. Previous studies of prostate cancer have not investigated the gene copy number or expression of *CDH7*, *CDH19* and *CDH20*, located on chromosome 18q22-q23 (Kools et al., 2000; Shimoyama et al., 2000). In normal cells, the expression of *CDH7* is restricted to the brain, prostate and testes (Kools et al., 2000). Although the thee genes, *CDH7*, *CDH19* and *CDH20*, are closely clustered in a region of about 5.7 Mb on chromosome 18q, the expression of each gene is quite variable in different tissues (Kools et al., 2000). From our analysis using quantitative PCR, it appears that CDH7 expression is increased up to eight-fold in prostate tumors, relative to adjacent normal prostate.

The type II cadherin family, to which *CDH7* belongs, differs from type I cadherins, including E-cadherin, N-cadherin and P-cadherin, by the absence of a histidine-alanine-valine (HAV) motif within the first cadherin repeat (Shimoyama et al., 2000). It has been shown that the lack of the HAV domain in type II cadherins does not compromise their ability to form cell-cell adhesion complexes (Shimoyama et al., 2000). The type I cadherins mainly show homotypic interaction when forming cell-cell adhesion (Mason et al., 2002). Unlike type I cadherins, type II cadherins, including *CDH7* and *CDH14*, have been shown to interact heterotypically in a cell aggregation assay using mouse fibroblasts that lack E-cadherin, which were transfected with various human type II cadherins (Nagafuchi et al. 1987). Currently, it is not known whether such heterotypic interactions occur *in vivo*, and what might be the consequences of these interactions. In our array CGH analyses, we did not find any copy number change for *CDH20*, which is located approximately 5 Mb proximal to *CDH7*. *CDH19*, which has a more ubiquitous pattern of expression (Kools et al., 2000), showed only low-level amplification in prostate tumors by array CGH.

The changes in the expression patterns of cadherins can contribute to the tumor progression by altering cell-matrix interactions (Cavallaro and Christofori, 2004). In prostate cancer, a transient downregulation of E-cadherin expression was noted in localized cancer, while the metastatic cancers showed normal expression (Rubin et al. 2001). A ‘switch’ in the expression pattern from a normal epithelial E-cadherin type to the mesenchymal N-cadherin has been shown in breast cancer (Hazan et al., 1997), prostate cancer (Tomita et al., 2000) and melanomas (Sanders et al., 1999). In prostate cancer cell lines, a complex pattern

of cadherin expression has been observed; the levels of each member, including E-cadherin, N-cadherin, P-cadherin, R-cadherin, K-cadherin and cadherin-11, have differing ratios in a particular cell line (Bussemakers et al., 2000). The influence of inappropriate expression of mesenchymal cadherins is exhibited by breast cancer cells, where overexpression of N-cadherin resulted in increased invasiveness and metastasis (Hazan et al., 1997). The loss of E-cadherin expression and concomitant overexpression of mesenchymal cadherins, N-cadherin and *CDH11* have been reported in high grade prostate tumors (Tomita et al., 2000). The mechanism by which N-cadherin and *CDH11* are upregulated in the tumors has not been studied. Interestingly, N-cadherin is located at chromosome 18q11. About 30% of 37 hormone-refractory prostate tumors exhibit gain of 18q12 by CGH (Nupponen et al., 1998). A mutually exclusive pattern of expression has been seen for *CDH7* and E-cadherin in human melanoma cell lines derived from tumor biopsies from patients with primary or metastatic melanoma (Moore et al., 2004). This study reported that normal melanocytes did not express *CDH7*, while melanoma cell lines expressing *CDH7* grew tumors in nude mice, thus indicating that *CDH7* plays a crucial role in melanoma tumorigenesis. Further exploration is needed to determine whether the upregulation of *CDH7* in prostate cancer interferes with normal cell-adhesion and signaling.

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Table 1. Prostate tumor samples with various pathological grades showing amplification at chromosome 18q22.1

Tumor	Age	Gleason	Stage	Amplification at 18q22.1
N10	66	6	T2CNXMX	Medium*
N12	64	6	T2CNXMX	Medium
N15	72	7	T2CNXMX	Medium
N22	59	7	T2CN0MX	High
N26	56	6	T2CNXMX	Low
N30	73	5	T2CNXMX	Medium
N31	72	6	T2CNXMX	Medium
N32	54	8	T2CN0MX	Low
N34	52	7	T3BN0MX	Normal
N35	56	7	T2CNXMX	Low
N36	62	6	T2CNXMX	Medium
N37	62	9	T3AN0MX	High
N38	59	6	T2CNXMX	Medium
N39	60	7	T3NXMX	Low
N40	70	7	T2CNXMX	High
N41	67	7	T3BN0MX	High
N42	67	8	T2CN0MX	Low
N43	65	5	T2BNXMX	Low
N44	53	7	T2BNXMX	Medium
N45	55	9	T2BN0MX	Low
N47	71	9	T3BN0MX	Low
N49	56	9	T2CNXMX	Low

* Log_2 ratio 1-1.5: Low; Log_2 ratio 1.5-2.0: Medium; Log_2 ratio >2.0: High

Titles and legends to figures

Fig. 1. Array CGH on prostate tumor samples showing amplification of the genomic region contained within RP11-775G23 at chromosome 18q22.1. Genomic DNA from prostate tumors was labeled with Cy5 in experiment number 1 (represented by blue curve) and normal male reference was labeled with Cy3. Dye-reversal was done for the same pair of DNAs in experiment number 2 (represented by red curve). The X-axis represents the cytogenetic position of each BAC clone (in Mb) and Y-axis represents $\log_2$ ratio of Cy5/Cy3.

Fig. 2. Validation of the amplification at chromosome 18q22.1 using FISH on paraffin-embedded tumor sections. A probe derived from BAC, RP11-775G23 (FITC) detects multiple copies of this region in the prostate tumors (a & b), while corresponding adjacent normal cells (e & f) show only two copies. The control probe (spectrum orange) was a human chromosome 18-specific centromeric probe. A probe derived from RP11-389J22 (FITC), the clone flanking RP11-775G23, also shows amplification in the tumors (c & d), while adjacent normal cells (g & h) show only two copies.

Fig.3. Identification of minimal region of amplification at chromosome 18q22.1. A probe proximal to RP11-775G23, RP11-453M23 (Spectrum Orange) is present in two copies in both tumor (a) and normal tissues (b), while a distal probe RP11-425M2 (FITC) is deleted in the tumor section (a). These two probes are present approximately 680 kb apart from each other on the chromosome.

Fig. 4. Real-time quantitative PCR analysis of *CDH7* in prostate tumor samples. a. *CDH7* expression in six different tumors relative to the prostate epithelial cell line, PZ-HPV-7. The expression level of *CDH7* in PZ-HPV-7 is considered as 1. The error bars depict the values for standard deviation. b. Relative expression levels of *CDH7* in four different tumors as compared to the matched adjacent normal tissue.

Figure 1

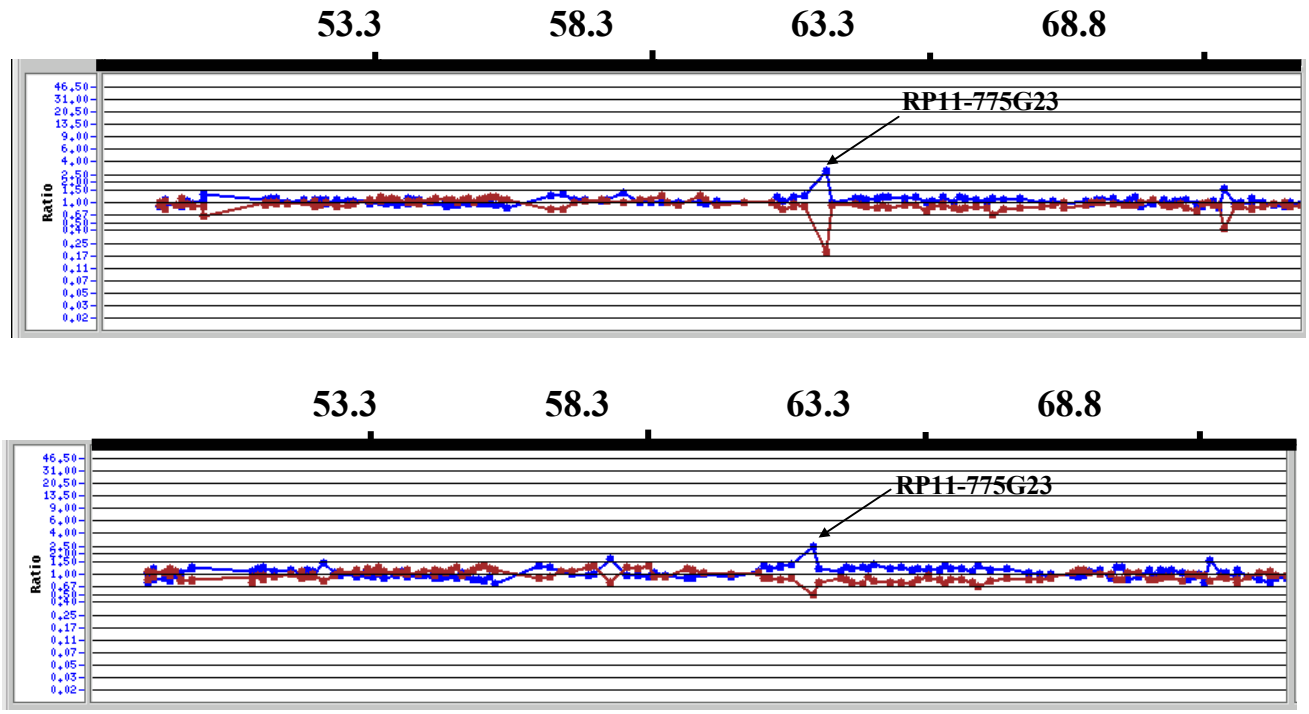

53.3
58.3
63.3
68.8
Ratio
RP11-775G23
53.3
58.3
63.3
68.8
Ratio
RP11-775G23

Figure 2

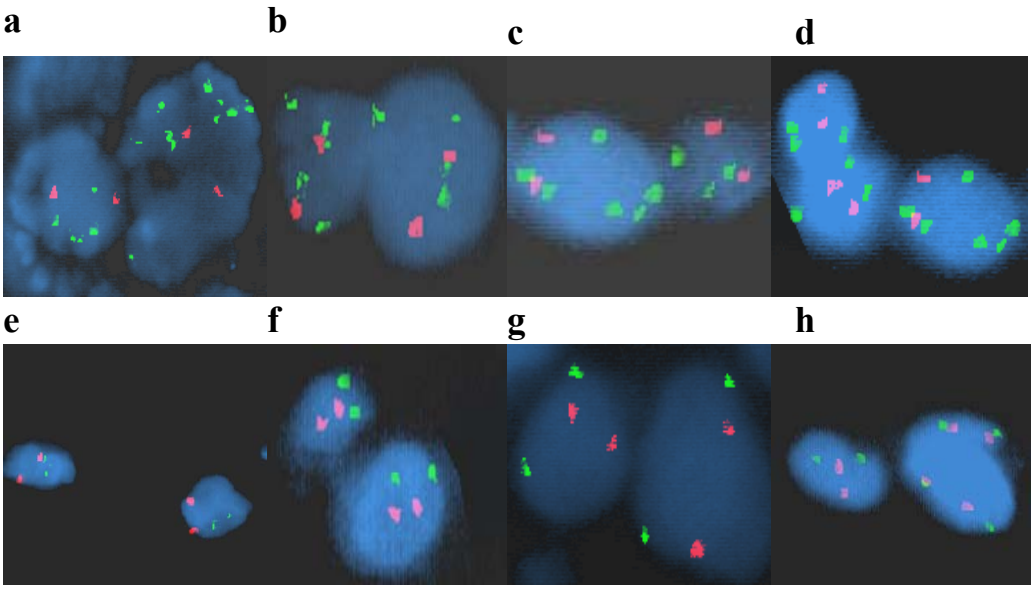


Figure 3

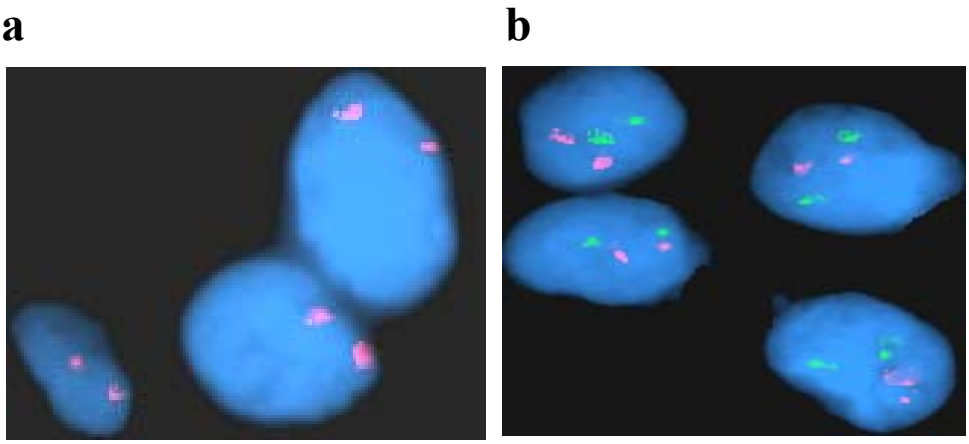
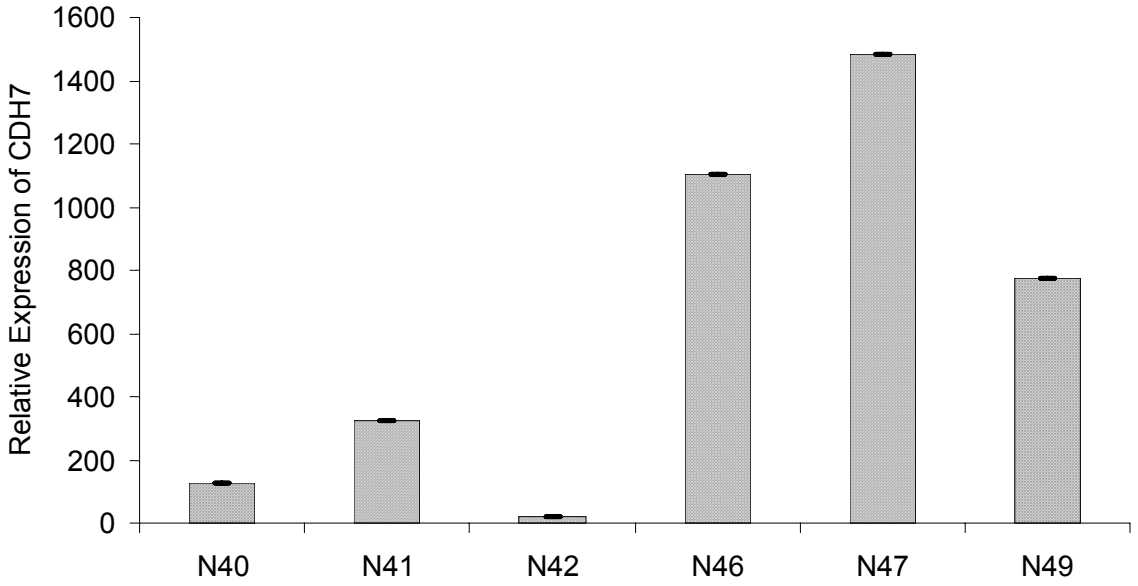
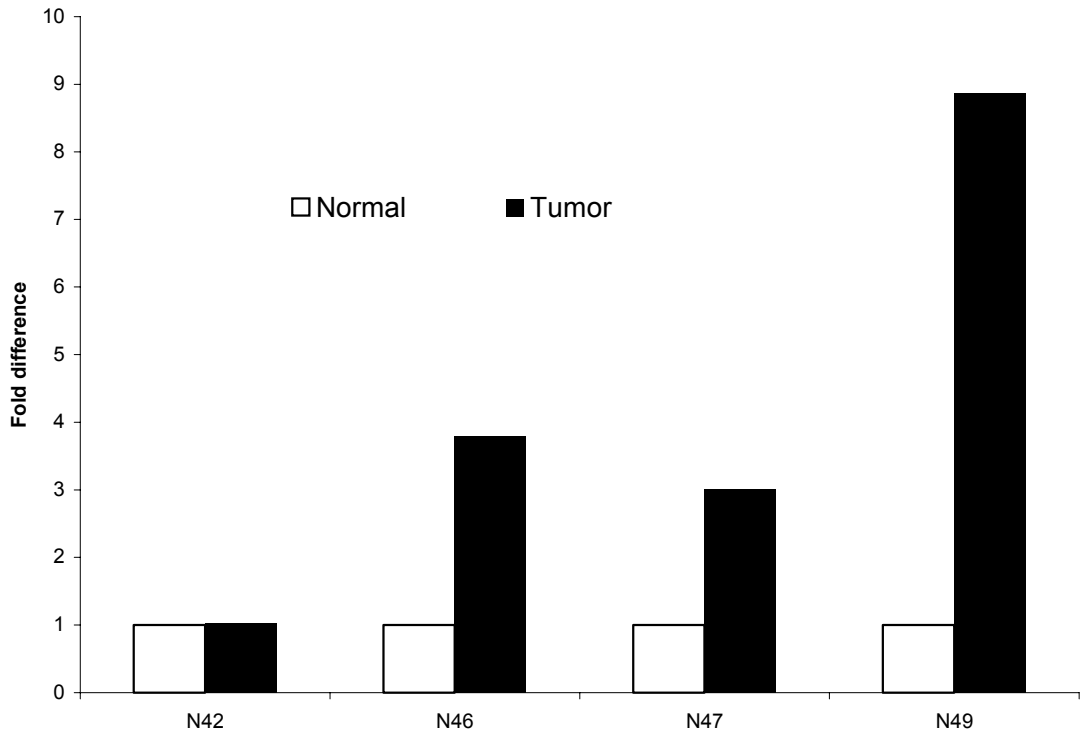


Figure 4

a


Relative Expression of CDH7
1600
1400
1200
1000
800
600
400
200
0
N40
N41
N42
N46
N47
N49

b


Fold difference
10
9
8
7
6
5
4
3
2
1
0
Normal
Tumor
N42
N46
N47
N49